Induced Pluripotent Stem Cell Transplantation in the Treatment of Porcine Chronic Myocardial Ischemia

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Background. This study was designed to test the effects of induced pluripotent stem cell (iPSC) in the treatment of chronic myocardial ischemia.

Methods. The reprogramming of passage 3 myocardial fibroblasts was performed by using the lentiviral vector containing 4 human factors: OCT4, SOX2, KLF4, and c-MYC. The iPSC colonies at P12-17 were allogeneically transplanted into ischemic myocardium of 10 swine by direct injection. Cohorts of 2 animals were sacrificed at 2, 4, 6, 8, and 12 weeks after injection.

Results. No signs of graft versus host disease were evident at any time points. At 2 weeks, clusters of SSEA-4-positive iPSCs were detected in the injected area. At 4 to 8 weeks, these cells started to proliferate into small spheres surrounded by thin capsules. At 12 weeks the cell clusters still existed, but decreased in size and numbers. The cells inside these masses were homogeneous with no sign of differentiation into any specific lineage. Increased smooth muscle actin or vWF positive cells were found inside and around the iPSC clusters, compared with non-injected areas. By real-time polymerase chain reaction, the levels of VEGF, basic FGF, and ANRT expression were significantly higher in the iPSC-treated myocardium compared with untreated areas. These results suggest that iPSCs contributed to angiogenesis.

Conclusions. Allogeneically transplanted pig iPSCs proliferated despite an ischemic environment in the first 2 months and survived for at least 3 months in immunocompetent hosts. Transplanted iPSCs were also proangiogenic and thus might have beneficial effects on the ischemic heart diseases.

(Marrow-derived stem or progenitor cells have been used for patients with ischemic myocardial diseases since 2003 [1–8]. Functional improvement has been reported by most clinical trials; however, no evidence has shown that marrow-derived cells can differentiate into beating myocytes in vitro, even though several studies claimed myocyte-like characteristics or markers after treating these cells with certain chemicals or factors [9–11]. Thus, most investigators believe that the beneficial improvement is mainly due to paracrine effects of implanted cells [12–16]. Induced pluripotent stem cells (iPSCs) have been recently generated in murine, human, and several other species since 2006 [17–19]. These iPSCs have become exciting tools for understanding the mechanisms of diseases and for potentially treating diseases through cell replacement therapy. In order to apply this new technology for future clinical use it needs to be tested in a large animal model. Recently, pig iPSCs have been derived from fetal fibroblasts by ectopic expression of 4 human transcriptional factors, OCT4, SOX2, KLF4, and c-MYC using lentiviral vectors [20]. These pig iPSCs showed pluripotency through in vitro differentiation assays and teratoma formation assays. However, these cells have failed to differentiate into beating myocytes in vitro. Recent studies have suggested that iPSCs seem to retain epigenetic memory because they have a higher tendency to differentiate into the lineages from which they were reprogrammed [21–23]. Therefore, we sought to derive iPSCs from porcine myocardial fibroblasts and hope these iPSCs can facilitate our efforts to generate cardiomyocytes. In this study we performed allogeneic transplantation of the cardiac fibroblast-derived pig iPSCs into chronic ischemic myocardium, and compared their effects with fetal fibroblast-derived iPSCs [20]. The results were compared with those using bone marrow-derived mesenchymal stem cell (MSC) previously reported by our laboratory [24, 25].

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Material and Methods

Pig Myocardium-Derived Fibroblasts Preparation and Culture

A tissue explant method was used for the myocardium-derived fibroblast culture. Briefly, a full length of left ventricular tissue (1 x 1 cm) was obtained from a healthy adult donor (Yorkshire, 3 months old). After washing 3 times in serum-free x 2 penicillin-streptomycin solution containing Dulbecco’s Modified Eagle Medium (DMEM), the tissue was cut into small pieces (2-mm cube) and then washed twice. The small tissue clumps were transferred into a 10-cm culture dish, and cultured with DMEM supplemented with 10% fetal bovine serum, x 1 penicillin-streptomycin solution. Fibroblasts began to migrate out of the tissue fragments and into the surrounding area after 3 to 7 days in culture. These fibroblasts were harvested by trypsinization and passaged in DMEM with 10% fetal bovine serum (Figs 1A–1D).

Pig iPSC Derivation and Culture

Passage 3 myocardium-derived fibroblasts were seeded in 6-well culture plates at a density of approximately 1 x 10^5 per well. On the next day they were transduced with StemCCA lentiviral vectors (Millipore, Billerica, MA) containing the 4 human factors, OCT4, SOX2, KLF4, and c-MYC, following the manufacturer provided protocol. After viral transduction, the fibroblasts were cultured in DMEM medium with 10% fetal bovine serum for 5 more days and then trypsinized and plated on 10-cm tissue culture dishes with irradiated mouse embryonic fibroblasts (MEF). The next morning, the culture medium was switched to standard human embryonic stem cell (ESC) medium and changed daily afterward. The iPSC-like colonies began to appear 5 days after switching to ES medium (Figs 1E, 1F). These colonies were manually picked about a week later into 24-well plates and subsequently passed and expanded following standard human ESC/iPSC cultural protocol. Passage 12–17 pig iPSCs were used for the allogeneic transplantation study.

Animals

Yorkshire domestic pigs, initially weighing 15 to 20 kg were used as allogeneic iPSC recipients for this study. The experimental protocol was approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute, and all procedures conformed to the Guide for the Care and Use of Laboratory Animals (National Academy Press, 1996, Washington, DC).

Ameroid Placement and iPSC Transplantation

To create a chronic ischemic model, all animals underwent ameroid constrictor placement around the proximal left circumflex coronary artery [26]. Four weeks later, a second left thoracotomy was performed on each animal;
the circumflex territory (ischemic zone) was exposed and injected with an average of \(1.6 \times 10^7\) iPSCs (from four 10-cm culture dishes with confluent iPSC colonies, suspended in 2.5 mL of normal saline, with 25 injection sites). Cohorts of 2 animals were sacrificed at 2, 4, 6, and 8 weeks, and 3 months after injection and the hearts were harvested and sectioned to study the differentiation of the injected cells.

To test if different stem cell lines behave differently in terms of their differentiation and proliferation capabilities, iPSCs kindly provided by Dr Robert’s laboratory were used for allogeneic transplantation using the same methods. Before injection, these cells were expanded using the methods suggested by the provider [20].

**Histologic and Immunohistochemistry Analysis**

Myocardial tissues with the iPSCs transplantation were cut into 5 \(\times\) 5 mm-thick pieces, either collected in cassettes and fixed with 10% buffered formalin for paraffin embedding or in OCT for frozen sections with no fixation. Lung, liver, kidney, and spleen samples were also collected for possible cell tracking. Paraffin-embedded sections were stained with hematoxylin and eosin for morphologic analysis. The immunofluorescent staining was performed using rabbit polyclonal antibody against human OCT4 and SSEA4 to detect pluripotent marker genes. Antibody against von Willebrand Factor (vWF) was used to detect vascular endothelial cells, and mouse monoclonal antibody against human smooth muscle actin to detect vascular smooth muscle cells. Mouse monoclonal antibody against human desmin was used to detect myocytes. All above mentioned antibodies were purchased from Dako North America (Carpinteria, CA). The incubations of primary antibodies were followed by detections of fluorescein isothiocyanate conjugated anti-mouse IgG or rhodamine conjugated anti-rabbit IgG and the nuclei were labeled with 4’,6-diamidino-2-phenylindole.

**Ribonucleic Acid Preparation and Real-Time Reverse-Transcription Polymerase Chain Reaction**

To test the different gene expression profiles of the tissues with or without iPSC injection, total RNAs from these samples were isolated using RNeasy Kit (Qiagen, Valencia, CA) for real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis. The first-strand cDNAs were synthesized from 500 ng of total RNA using a SuperScript III kit (Invitrogen Life Technologies, Carlsbad, CA) and random hexamers. The cDNA templates were then mixed with PCR reaction solution containing 100 nM of both forward and reverse primers, Universal PCR master mix with SYBR Green 1 (QuantiTect SYBR Green RT-PCR Kit, QIAGEN Inc). The PCR amplification was performed on a multicolor qRT-PCR detection system (IQ5, Bio-Rad Lab, Inc, Hercules, CA) as follows: 10 min of initial denaturation at 95°C followed by 45 cycles of 15 sec. of denaturation at 95°C and 1 minute of annealing/extension at 60°C. Each sample
Fig 3. Allogeneic transplantation of myocardial fibroblast-derived induced pluripotent stem cells (iPSCs). (A) Arrow indicating an area with 3 spherical shaped tumors at 4 weeks after injection (×12.5); (B) and (C) higher magnification of (A) (×100 and ×200); (D) arrow indicating an area with 3 irregular shaped tumors at 6 weeks after injection (×12.5); (E) and (F) higher magnification of (D) (×100); (G) and (H) positive stage-specific embryonic antigen-4 staining (red) of the tumor-like cell clumps at 6 weeks (×400); (I) arrowhead indicating positive OCT 4 (green) and arrow indicating smooth muscle actin staining (red) (×400); (J)–(L) arrows indicating cell clumps were also found in lung (J and K) and spleen (L) at 6 weeks similar to those found in ischemic heart area (×100); (M) arrow indicating a normal splenic nodule with a central artery in the center which was not found in iPSC formed tumors (×400); (N)–(R) arrows indicating two sets of photos showing similar spherical-shaped small tumors from 2 different animals at 8 weeks. (N) and (P) = ×12.5; (O), (Q), and (R) = ×100.
was analyzed in duplicates. Three qRT-PCR reactions were performed for the examination of each signal gene. For normalization, the average of the expression level of two housekeeping genes, swine GAPDH and β2-microglobulin were used. Analysis of relative quantification was performed as previously described [27]. The sequences of PCR primers are listed as following:

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences</th>
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<tbody>
<tr>
<td>FGF F</td>
<td>GGA GAA GAG CGA CCC TCA CA</td>
</tr>
<tr>
<td>FGF R</td>
<td>CGG TTT GCA CAC ACT CCT TTG</td>
</tr>
<tr>
<td>VEGF F</td>
<td>CGC CAT GCA GAT TAT GCG GAT C</td>
</tr>
<tr>
<td>VEGF R</td>
<td>ACT CAA GCT GCC TCG CCT TGC A</td>
</tr>
<tr>
<td>ARNT (HIF1B) F</td>
<td>AGC CAT TGT TCA GAG GGC TA</td>
</tr>
<tr>
<td>ARNT (HIF1B) R</td>
<td>CCG CCG TTC AAT TTC ACT AT</td>
</tr>
<tr>
<td>OCT 3/4 F</td>
<td>AGT GAG AGG CAA CCT GGA GA</td>
</tr>
<tr>
<td>OCT 3/4 R</td>
<td>TCG TTG CGA ATA GTC ACT GC</td>
</tr>
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Results

Pluripotency Tests of Cardiac Fibroblast-Derived iPSCs

After 5 passages in cultures on MEF feeder layers, the colonies became uniform in appearance with morphology resembling mature human iPSC colonies (Figs 1G, 1H). They were positive for live staining with Tra-1-60 and Tra-1-81 antibodies. They were also positive for alkaline phosphatase staining, as well as SSEA-4, OCT4, Nanog, and Sox2 antibody staining (Figs 2A–2G). After culturing selected large colonies for 4 weeks in ultralow attachment plates, embryoid bodies containing the typical 3 differentiated germ layers were found. After injecting into immunocompromised mice, these cells formed teratomas. These cells can also differentiate into neuronal lineages as evidenced by strong staining of Tuj-1 (Figs 2H–2I). After culturing selected large colonies for 7 days in ultralow attachment plates and reseeding on MEF feeder layers, positive GATA-4 staining were found in some of the reseeded cells.

Statistical Analysis

Data are presented as means ± standard deviation. The standard Student t test was used for gene expression comparison and a p value of less than 0.05 was considered significant.

Animal Outcome

After transplantation, there were no signs of adverse side effects or graft versus host disease. Three unexpected animal deaths were caused by severe surgical wound infection or pulmonary edema after ameroid placement.

Fig 4. (A) Smooth muscle actin (red) and von Willebrand Factor (green) staining at 8 weeks; arrow indicating a cell clump; (B) negative control of (A); (C) staining of (A) at 4 weeks; arrow indicating a cell clump; (D) staining of (A) at noninjected area at 4 weeks after injection. Magnifications (A)–(D) = ×100.
determined by autopsy examination and not as a result of cell injections.

**Histology Analysis**

Two weeks after cell injection, clusters of SSEA-4 positive cells were detectable in the frozen sections of the injected area using immunofluorescent staining. However, these cells could hardly be seen in hematoxylin and eosin stained paraffin sections. This could be resulted from the small number (one tenth) of injected iPSCs as compared with previous MSC studies. Quite to our surprise, at 4 to 8 weeks posttransplantation these clusters of cells started to proliferate into spherical shaped small tumors surrounded by thin layered capsules within the injected area (Figs 3A-3R). The cells inside these tumors showed a homogeneous phenotype with no signs of differentiation into any lineages. Most of these tiny tumors were spherical shaped (Figs 3B, 3C, 3R) and some of them were irregular or elongated (Figs 3E, 3N, 3O), but all had thin layers of capsules. There were multiple spherical shaped tumors within an elongated cell cluster (Fig 3O), suggesting variable growth speed within a single cell cluster. All these small tumors stained positive for OCT4 and SSEA4 suggesting these cells were injected iPSCs (Figs 3G-3I). Interestingly, 3 months after injection, the small tumors showed no sign of further growth. Instead, these small tumors became smaller and irregular compared with those seen in the 4-week to 8-week time points. Similar strong SSEA4 positive cell clusters were found in the injected area but in smaller numbers. These results suggested that myocardium-derived iPSCs survived inside the chronic ischemic environment after allogeneic transplantation and started to proliferate after about 2 weeks. Growth was limited in the immunocompetent host, evidenced by the fact that the small tumors found in the 4-week to 8-week time points were no longer becoming larger at the 12-week time point. In MSC-injected animals, cell clusters were also clearly found in the injected area. However, there were more cells in the early time points and the number of MSC decreased gradually over the 3-month period postinjection. Additionally, there was no sign of cell proliferation or evidence of capsules around the MSC clusters.

**Angiogenesis Related Tests**

At the 4 and 8 week time points, immunofluorescent staining showed increased smooth muscle actin and vWF positive cells inside and around the iPSC clusters (Figs 4A-4C) compared with those in noninjected areas (Fig 4D). This finding may suggest that injected iPSCs are associated with angiogenesis. No desmin positive cells were found within or around these cell clusters. The expression of VEGF, FGF, and ANRT, as assessed by qRT-PCR, were significantly higher in the injected myocardium compared with noninjected myocardium.
both in the 4-week and 3-month time points (Fig 5). These results suggested that injected iPSCs might have contributed to the formation of new blood vessels to a comparable level as those injected with MSCs. This angiogenic effect may have occurred as a result of paracrine-like secretion of pro-angiogenesis factors. This finding suggests a beneficial effect of the iPSC transplantation even though injected iPSCs were not sufficient enough to regenerate the damaged heart.

**Allogeneic Transplantation of Another iPSC Cell Line**

Similar results were found from the animals that received another strain of transplanted iPSCs. Spherical and irregular shaped small tumors were found in 4-week to 8-week time points and the sizes decreased over 3 months. The small tumors stained positive for OCT4 and SSEA4, suggesting that injected iPSCs may keep their pluripotency markers but not differentiate in other cell lineages. Figure 6 (A-H) shows the histology images with a larger amount of small tumors in the 8-week time point, which dramatically decrease in the 3-month time point.

**Comment**

We report the first, to our knowledge, in vivo iPSC allogeneic transplantation into the ischemic myocardial environment of a large animal model that focuses on the fate and differentiation abilities of the transplanted cells in the United States. We expected myocardium-derived iPSCs developed in our laboratory would preferentially differentiate into the myocyte lineage compared with those derived from skin, blood, or other non-muscle lineage cells. The myocardium-derived iPSCs carry myocardial epigenetic memory, which most investigators believe will influence their differentiation capabilities [21–23]. Unfortunately, our data did not support this expectation. Rather, the iPSCs derived from pig myocardium only showed limited myocyte differentiation as we found only a few GATA4-positive cells in dissociated embryoid bodies. No beating myocytes were found when various cultural protocols were performed. The iPSC line provided by Dr Roberts’ group, which was derived from porcine fetal fibroblasts, also lacked the ability to differentiate into beating myocytes in our hands. The underlying reason for this phenomenon is unclear, but one possibility is that the lentiviral vectors with the 4 reprogramming genes, which are integrated into the genome, are not completely silenced. The residual levels of these transcription factors may hinder the efficient differentiation into cardiac lineages of the injected iPSCs.

Even though both lines of iPSCs failed to show any signs of differentiation when transplanted into ischemic myocardium by direct injection, this study did provide us with several important findings: (1) when allogeneically transplanted, the iPSCs proliferated in the early stage, reached a peak level at 8-weeks posttransplantation, then gradually declined in cell number; (2) once transplanted, iPSCs showed proangiogenic effects, potentially as a result of paracrine-like secretion of factors which enhanced the proangiogenic signal pathways of VEGF, FGF, and ANRT; (3) compared with our previous study on MSCs transplantation in the same porcine model, they both showed beneficial effects on angiogenesis, but iPSCs seemed to possess a greater aptitude toward survival and proliferation; (4) the recipient pigs were generally healthy and there were no signs of deleterious tumor formation. However, we did find tumor-like cell clumps in the lung and spleen of 1 animal at the 6-week time point, suggesting that the injected iPSCs can survive and proliferate in organs other than the heart. This is most likely caused by accidental cell leaking into the blood stream at the time of injection rather than metastasis. Therefore, extra caution must be exercised to avoid leakage when performing direct injection in future clinical cell-based therapy. The consequences for the leaked iPSCs are not yet clear. More detailed long-term studies are needed to investigate whether they will interfere with the function of other organs and whether they will form teratomas, although most of these leaked cells appeared to be short lived. The current study terminates at 3 months post-transplantation. It is very useful for quickly assessing the survival and differentiation potential of iPSCs in vivo, but long-term observation and analysis are needed to unequivocally demonstrate whether this method is safe enough for clinical use.

In summary, in an ischemic environment pig iPSCs can continue to proliferate in vivo for 2 months after injection. However, the proliferation ability of the iPSCs was limited within the immunocompetent hosts. The iPSCs we developed from pig myocardial fibroblasts showed limited ability to differentiate into beating myocytes. For future cell-based therapy, iPSCs with a higher propensity to differentiate into cardiomyocytes will be ideal for assessing their potential for treating myocardial infarction. We are planning to use more efficient but non-integrative vectors for reprogramming cardiac fibroblasts into iPSCs.

The authors are particularly grateful to Dr R. Michael Roberts and his colleagues for providing them with the porcine fetal fibroblast-derived iPSCs.

**References**


